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PCT/GB00/00606

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

WHITAKER, Iain, Mark
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ROYAUME-UNI

Date of mailing (day/month/year)
02 November 2000 (02.11.00)

Applicant's or agent's file reference
PA 3442 PCT INT

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/00606

International filing date (day/month/year)
21 February 2000 (21.02.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

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2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

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3. Further observations, if necessary:
The above-mentioned inventor is to be considered as applicant/inventor for US only, since he assigned his rights for all designated States except US to a new applicant as indicated below.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
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Authorized officer
Dominique DELMAS
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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
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Washington, D.C.20231
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in its capacity as elected Office

Date of mailing (day/month/year) 13 October 2000 (13.10.00)	
International application No. PCT/GB00/00606	**Applicant's or agent's file reference** PA 3442 PCT INT
International filing date (day/month/year) 21 February 2000 (21.02.00)	**Priority date** (day/month/year) 20 February 1999 (20.02.99)
Applicant HARBRON, Stuart	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 September 2000 (15.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PA 3442 PCT INT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00606	21/02/2000	20/02/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/34		
Applicant ZETATRONICS LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/09/2000	29.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Luzzatto, E Telephone No. +49 89 2399 8169

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00606

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1,3-6,8,9	as originally filed			
2,2a,7	as received on	07/02/2001	with letter of	26/01/2001

Claims, No.:

1-25	as received on	07/02/2001	with letter of	26/01/2001

Drawings, sheets:

1/6-6/6	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-25
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

PART V

1) None of the available documents discloses a method as that of independent claims 1, 12 and 13 or a kit as that of independent claim 16. Hence these claims, as well as claims 2-11, 14, 15 and 17-25 are novel (Art. 33(2) PCT).

2) D1 is considered to represent the closest prior art. It discloses a method for detecting ligand binding reactions comprising the use of a nuclease as a label. The nuclease cleaves a compound of formula RpX (where RpX has the same meaning as in present claim 1) to produce Rp and X-H; X-H is then detected. Ribonuclease S is the exemplified enzyme.
The subject-matter of claim 1 differs from D1 in that it relates to the use of an enzyme capable of cleaving RpX so as to produce R-H and pX, whereby pX is then detected (if R= 3' nicotinamide derivative, then either pX or R-H can be detected).
The technical problem underlying the present invention may thus be seen in the provision of an alternative assay to that of D1.
None of the documents cited in the Search Report could be combined with D1 so as to allow the obtention in an obvoius way of the method of claim 1: they either relate to methods comprising the detection of the R moiety (and not disclosing the use of nicotinamide derivatives) (D2-D5) or relate to the use of NAD(2)P for the detection of e.g. phosphatases (D6). Moreover, neither of them relates to a method for the detection of binding events between specific binding pairs.
An inventive step should thus be acknowledged for independent claims 1, 12 and 13 and for claims 2-11, 14 and 15 dependent thereon.

3) D5 discloses a method for the detection of nuclease activity comprising the use of compounds of formula RpX (see fig. 1), which are hydrolysed to give R (adenosine) and pX (phenylphosphonate). The former is then detected.
D5, therefore, does not render obvious the subject-matter of claim 16 because it does not provide any incentive that would have lead the skilled person to combine in a kit the RpX compound with a system for the detection of pX instead of adenosine.
None of the other available documents relates to a method comprising the detection of pX, and thus cannot render obvious the subject-matter of claim 16

(see however item VIII.1).
Also claims 16-25, therefore, appear to meet the requirements of Art. 33(3) PCT.

4) Claims 1-25 relate to industrially applicable subject-matter (Art. 33(4) PCT.

PART VIII

1) The expression "detection system" to be found in claim 16 is devoid of a clear technical meaning in the context of the claim. Hence the said claim 16 as well as claims 17-25 dependent thereon are unclear (Art. 6 PCT).

PCT D INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETERMINATION OF NUCLEASE ACTIVITY

(57) Abstract

A method for detecting a nuclease enzyme is disclosed which comprises a method for detecting a nuclease enzyme comprising the steps: a) contacting said enzyme with a compound of formula RpX, wherein R is a 3' nucleosidyl derivative, p is a phospho radical, and X is an esterifiable moiety or, only if R is a 3' nicotinamide derivative, X is an esterifiable moiety or H, whereby ROH and pX are produced, and b) detecting said pX moiety or, only if R is a 3' nicotinamide derivative, detecting the pX moiety or the ROH moiety. In preferred embodiments the invention provides a method for detecting a nuclease enzyme that is free in solution, immobilised on a surface, or attached to a member of a specific binding pair. The method of the invention may thus be applied as a detection step in nucleic acid hybridisation assays, enzyme immunoassays and ligand:receptor binding assays. The invention provides a variety of methods for detecting the detectable moieties produced. These include fluorometric, colorimetric, and luminometric endpoints. Enzyme cycling and apoenzyme reactivation assays are also provided.

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Det rmination of Nuclease Activity

The present invention is concerned with a method and kit for detecting the presence of a nuclease enzyme.

Self (EP0027036A, EP0049606A, EP0058539A, EP0060123A, US4446231, US4595655, US4598042, and US4769321) discloses methods for detecting phosphatase enzymes that produce NAD or NADH from NADP or NADPH respectively.

Akihiro (US5589349) discloses the use of enzymes with improved stability in a cycling assay for alkaline phosphatase.

Fisher et al disclose the assay of nucleases using FADP as a substrate (WO98/19168A).

Harbron et al (Analytical Biochemistry (1991) **198**:47-51) disclose an assay for alkaline phosphatase which relies on the production of FMN, which is detected using apoglycolate oxidase.

Harbron et al (Journal of Bioluminescence and Chemiluminescence (1991) **6**:251-258) disclose the luminometric detection of alkaline phosphatase based on the production of FMN, which is detected using the bacterial bioluminescent system.

Harbron (GB2324370B) discloses the use of nuclease P1 in a nucleic acid hybridisation assay in which excess probe is destroyed.

Stanley (Methods in Enzymology (1978) **57**:215-223) discloses the quantitation of NADH, NADPH and FMN using bacterial luciferase.

Rabin et al. (US4745054) discloses prosthetogenic enzyme amplification assays in which a pyrimidine ribonucleoside 3′-phosphate ester RpX is hydrolysed by ribonuclease to give XOH. XOH is a prosthetic group or a prosthetic group precursor such as thiamine, riboflavin, pyridoxal or pyridoxamine.

A number of patents assigned to Tropix describe 1,2-dioxetane derivatives of utility in chemiluminometric detection (US5869705, US5869699, US5866389, US5856522, US5851771, US5847161, US5840919, US5783381, US5777133, US5763681, US5756770, US5707559, US5679803, US5679802, US5652345, US5639907, US5637747, US5625077, US5605795, US5538847, US5397852, US5342966, US5330900, US5326882, US5225584, US5220005, US4978614, US4956477, US4931569, US4931223, US5843681, US5831102, US5773628, US5591591, US5582980, US5543295, US5145772, and US4952707).

The above citations are included herein by reference in their entirety.

Broadly, the present invention discloses in a first aspect a method for detecting a nuclease enzyme which comprises the steps of:

a) contacting said enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is an esterifiable moiety or, only if R is a 3' nicotinamide derivative, X is an esterifiable moiety or H, whereby ROH and pX are produced; and

b) detecting said pX moiety or, only if R is a 3' nicotinamide derivative, detecting the pX moiety or the ROH moiety.

In one preferred embodiment, the present invention discloses a method for detecting a nuclease enzyme which comprises contacting said nuclease enzyme with a compound of formula RpX, wherein R is a 3´nicotinamide derivative, p is a phospho radical, and X is H or an esterifiable moiety, whereby ROH and pX are produced, and detecting said ROH moiety.

In its embodiments, the invention may provide a method for detecting a nuclease enzyme that is free in solution, immobilised on a surface, or attached to a member of a specific binding pair. The method of the invention may thus be applied as a detection step in nucleic acid hybridisation assays, enzyme immunoassays and ligand:receptor binding assays.

In preferred embodiments the invention provides a variety of methods for detecting the detectable moieties produced. These include fluorometric, colorimetric, and luminometric endpoints. Enzyme cycling and apoenzyme reactivation assays are also provided.

In a further aspect the invention provides a kit for carrying out the method.

Preferred embodiments of the invention may enable one to achieve one or more of the following objects and advantages:

(a) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields products that are easily detected. An advantage of the present invention is that the assay may be easily performed using equipment commonly available in the laboratory.

(b) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields a product that is a prosthetic groups for an enzyme. An advantage of the present invention is that the assay is rapid and/or has high sensitivity.

(c) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields a product that can be detected by chemiluminescent or bioluminescent means. An advantage of the present invention is that the assay is rapid and/or has high sensitivity.

(d) to provide a method to detect a complex formed between two members of a specific binding pair, in which one of said members is labelled with a nuclease enzyme. An advantage of the present invention is that the complex may be rapidly and/or sensitively detected.

(e) to provide a kit for carrying out the method of the invention.

Some embodiments of the invention will be described in more detail, by way of example, with reference to the accompanying drawings in which:

Figure 1 is a diagrammatic representation of the conversion of NAD3P to NAD through the action of a nuclease enzyme, and subsequent cycling of the NAD produced through the action of a dehyrogenase and a diaphorase enzyme, to produce a coloured formazan.

Figure 2 is a diagrammatic representation of the hydrolysis of a substrate by a nuclease enzyme to yield products for detection.

Figure 3 is a diagrammatic representation of the hydrolysis of a adenosine-3'-phosphoriboflavin derivative by the action of a nuclease enzyme to yield FMN, and subsequent reconstitution of an apoenzyme by the FMN to yield holoenzyme for detection.

Figure 4 is a diagrammatic representation of the hydrolysis of a nucleoside-3'-phospho-1,2-dioxetane derivative to yield the corresponding 1,2-dioxetane phosphate. This latter is converted to 1,2-dioxetane, which decomposes producing light.

Figure 5 is a standard curve for the detection of nuclease P1 in a NAD-NADH cycling reaction. The absorbance produced after 800 sec at different pH values is plotted against the amount of nuclease P1 present in the reaction mixture.

Figure 6 is a standard curve for the detection of FMN in an apoenzyme reconstitution assay. The absorbance produced at 324 nm is plotted against the concentration of FMN in an aliquot added to the reaction mixture for two different apoenzyme preparations: circle is sugar beet, triangle is spinach.

The present invention provides a method for detecting a nuclease enzyme.

The present invention provides a variety of methods for detecting ROH or pX. These approaches may be colorimetric, fluorimetric, or luminometric, or may be through enzyme cycling reactions or apoenzyme reactivation assays.

In a preferred embodiment, the substrate RpX is NAD3´phosphate (NAD3P). This differs from commonly occurring NADP, which carries the phosphate moiety at the 2' position. This is hydrolysed by a nuclease to give NAD, which may be easily and sensitively detected, either by spectrophotometry or fluorimetry, or by coupling with a bacterial luminescence system to produce light, as disclosed by Stanley, or through enzyme cycling, as disclosed by Self. In a preferred embodiment, NAD is converted to NADH through the action of a dehydrogenase enzyme. The dehydrogenase enzyme may be alcohol dehydrogenase or lactate dehydrogenase. The presence of NADH may be detected spectrophotometrically or fluorometrically. Referring to Fig. 1, which shows a particularly preferred embodiment, NAD3P is hydrolysed to give NAD. NAD is converted to NADH through the action of a dehydrogenase enzyme. A diaphorase enzyme reduces a tetrazolium compound, such as INT, to give NAD and a coloured formazan, the absorption of which can be measured at 492nm. The NAD produced may then be cycled back to NADH through the action of the dehydrogenase enzyme, leading to an ever-increasing rate of colour development. A similar approach may be used using NAD3PH as the substrate, which yields NADH on hydrolysis. In this embodiment, the NADH enters the cycle as a substrate for diaphorase.

Referring now to Fig. 2, which shows another preferred embodiment, the substrate is a nucleosidyl-3´-phosphodiester wherein X is, for example, riboflavin, thiamine, pyridoxamine or pyridoxal, B is a base, for example adenine, guanine, uracil, thymine or cytosine, or a derivative thereof, and R' is H or other substituent. These are hydrolysed by the nuclease enzyme to yield, for example, riboflavin phosphate (FMN), thiamine phosphate, pyridoxamine phosphate or pyridoxal

phosphate, respectively. These may be detected using an apoenzyme reactivation assay of the type disclosed by Rabin. For example, FMN may be detected using apoglycolate oxidase; pyridoxal phosphate may be detected using apoaminoacid transferase. In a particularly preferred embodiment, shown in Fig. 3, the substrate adenosine-3'-phosphoriboflavin wherein A is adenine and R' is H. This compound is hydrolysed by the nuclease enzyme to yield adenosine and FMN (riboflavin phosphate). FMN may be sensitively detected using an apoenzyme, such as apoglycolate oxidase, as described by Harbron et al. (Analytical Biochemistry (1991) **198**:47-51), or by bioluminescent detection, as described by Harbron et al. (Journal of Bioluminescence and Chemiluminescence (1991) **6**:251-258). In an analogous fashion, RpX may be adenosine-3'-phosphothiamine, adenosine-3'-phosphopyridoxamine or adenosine-3'-phosphopyridoxal, which upon hydrolysis yield thiamine phosphate, pyridoxamine phosphate or pyridoxal phosphate. These two may be sensitively detected using the corresponding; for example, glycolate oxidase, or a transaminases.

In a further preferred embodiment, illustrated in Figure 4, the substrate is a nucleosidyl-3′-phospho-1,2-dioxetane derivative, such as a nucleosidyl-3′-phosphoadamantyl derivative, wherein B is a base, for example adenine, guanine, uracil, thymine or cytosine, or a derivative thereof, and R' is H or other substituent. This is hydrolysed to yield an adamantyl phosphate derivative, which may be hydrolysed chemically or in the presence of a phosphatase enzyme to yield the corresponding adamantyl derivative, which decomposes chemiluminometrically. A further preferred embodiment utilises an adenosine-3'-phosphoadamantyl derivative, which, upon hydrolysis, yields an adamantyl-phosphate derivative. This may then be dephosphorylated by means of a phosphatase enzyme or chemically. For example, the adamantyl-phosphate derivative produced may be CDP-*Star* (R) from Tropix Inc. Upon dephosphorylation of CDP-*Star* (R) substrate by alkaline

phosphatase, a metastable chlorophenolate dioxetane anion intermediate is formed which decomposes and emits light at a maximum wavelength of 466 nm. A delay in reaching maximum light emission results since the dioxetane anion has a half-life of less than one minute to several hours, depending on the surrounding environment. Film or simple instrumentation may be used to quantitate the chemiluminescent signal, which is produced as a continuous glow due to the reaction kinetics of the system.

The nuclease enzyme is any enzyme that cleaves the substrate RpX to yield R and pX. In one embodiment the nuclease enzyme is an enzyme of class EC.3.1.30.1. In a preferred embodiment the nuclease enzyme is nuclease P1, nuclease S1 or mung bean nuclease. In a particularly preferred embodiment the nuclease enzyme is nuclease P1.

In one embodiment the nuclease enzyme is free in solution. In another embodiment the nuclease enzyme is immobilised on a surface. In further embodiments the nuclease enzyme is attached to one member of a specific binding pair.

The present invention provides a method for detecting binding events between specific binding pairs, in which one of the pair is labelled with the nuclease enzyme. The covalent attachment of the nuclease enzyme to this moiety is described in Fisher et al. (WO98/19168) and Harbron (GB2324370B), and may be achieved by a number of well-known methods using a wide range of heterobifunctional reagents. For example, the method of Carlsson *et al.* (*Biochem J* (1978) **173**: 723 - 737) may be used: the nuclease enzyme is reacted with 3-[(2)-pyridyldithio]propionic acid N-hydroxysuccinimide ester (SPDP) to give a 2-pyridyl disulphide-activated label. This allows disulphide exchange with a specific binding partner having a sulphydryl

group to yield a labelled specific binding partner. Other approaches for labelling the specific binding partner will be apparent to one skilled in the art.

In one embodiment the specific binding pair comprises an antibody and a hapten or antigen. In another embodiment the specific binding pair comprises a nucleic acid probe and its corresponding target sequence. In a further embodiment the specific binding pair comprises a biotin derivative and avidin, streptavidin or neutravidin. In a yet further embodiment the specific binding pair comprises a ligand and a receptor.

Thus the invention may be used to detect binding events in nucleic acid hybridisation assays, enzyme immunoassays, and receptor:ligand binding assays.

The present invention provides a kit for carrying out the method of the invention. The kit comprises a compound of formula RpX, and a detection system for detecting ROH or pX. In one embodiment, RpX is NAD3P

The following examples illustrate aspects of the invention, and are not intended to limit the scope of the invention.

EXAMPLE 1 – Assay of Nuclease P1

A premix containing the following reagents was prepared prior to the assay and stored at 4°C until required: 50l 0 .5 M citrate buffer, pH 6.3; 100l 10 m M INT; 10 l 5 mM NAD3'P; 10l ethanol; 30l diaphorase solution (30 U/ml); 10l alcohol dehydrogenase solution (3mg/ml) and 780 l of water.

10 l aliqouts from a serially diluted solution of nuclease P1 were dispensed into the wells of a microtitre plate. 90l of the premix were then added, and the plate incubated at room temperature. The change in absorbance at 490 nm was followed by means of a plate reader.

The performance of the assay is illustrated in Figure 5, which shows a standard curve for the assay of nuclease P1 using the above assay at pH 6.3, and at pH 6.0 and 6.7.

EXAMPLE 2 – Apoenzyme Reactivation Assay for the Detection of FMN

Apoglycolate oxidase was prepared as described by Harbron et al. (Analytical Biochemistry (1991) **198**:47-51). A standard curve for the estimation of FMN, shown in Fig. 6, was prepared as follows: 50 mM Tris-HCl buffer, pH8.3, 44 mU apoglycolate oxidase and 0.02 to 200M F M N in a total volume of 0.05 mL was incubated for 1h at room temperature. This was then added to 0.95 mL of 50 mM Tris-HCl buffer, pH8.3, containing 3.47 mM phenylhydrazine and 5.26 mM glycolic acid, and the linear rate of absorbance was measured at 324 nm.

Claims

1. A method for detecting a nuclease enzyme comprising the steps of:

   a) contacting the enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is an esterifiable moiety or, only if R is a 3' nicotinamide derivative, X is an esterifiable moiety or H, whereby ROH and pX are produced, and

   b) detecting said pX moiety or, only if R is a 3' nicotinamide derivative, detecting the pX moiety or the ROH moiety.

2. The method of claim 1 wherein pX is a prosthetic group.

3. The method of claim 2 wherein said prosthetic group is selected from the group consisting of: riboflavin 5'phosphate, pyridoxal phosphate, pyridoxamine phosphate and thiamine pyrophosphate or a derivative of any of them.

4. The method of claim 1, 2 or 3 wherein said 3'nucleoside is selected from the group consisting of adenosine, cytosine, guanine, thymidine and uridine or a derivative of any of them.

5. The method of claim 2 and optionally also 3 and/or 4 wherein said detecting step comprises contacting said prosthetic group with an apoenzyme.

6. The method of claim 5 wherein said apoenzyme is apoglycolate oxidase or a transaminase.

7. The method of claim 1 wherein X is a 1,2-dioxetane compound.

8. The method of claim 7 wherein said detecting step comprises contacting said 1,2-dioxetane phosphate with a phosphatase enzyme, whereby light is produced, and detecting the light produced.

9. A method for detecting a nuclease enzyme comprising the steps of:

   a) contacting said enzyme with a phosphodiester comprising a prosthetic group and a 3'nucleoside, whereby said prosthetic group is produced, and

   b) detecting said prosthetic group.

10. A method for detecting a nuclease enzyme comprising the steps:

a) contacting said enzyme with a compound of formula RpX, wherein R is a 3'nicotinamide derivative, p is a phospho radical, and X is an esterifiable moiety, whereby ROH and pX are produced, and

b) detecting said ROH moiety.

11. The method of claim 10 wherein said nicotinamide derivative is NAD or NADH.

12. The method of claim 10 or 11 wherein said detecting step comprises conducting enzymatic cycling of NAD-NADH interconversions in the presence of a dehydrogenase, a substrate for said dehydrogenase, a tetrazolium dye and a diaphorase, and detecting the amount of the NAD or NADH with a colour-development signal of formazan which is produced by the action of diaphorase and NADH-NAD conversions.

13. A kit for detecting the presence of a nuclease enzyme comprising:

(a) a compound of formula RpX, wherein R is a 3'nucleosidyl derivative, p is a phospho radical, and X is an esterifiable moiety or, only if R is a 3' nicotinamide derivative, X is an esterifiable moiety or H, whereby ROH and pX are produced and

(b) a detection system for detecting pX or, only if R is a 3' nicotinamide derivative, a detection system for detecting the pX moiety or for detecting the ROH moiety.

14. The kit of claim 13 wherein RpX is NAD3P or NAD3PH.

15. The kit of claim 13 wherein RpX is a nucleoside-3'phosphoriboflavin derivative.

16. The kit of claim 13 wherein RpX is a nucleoside-3'-phospho-pyridoxal derivative.

17. The kit of claim 13 wherein RpX is a nucleoside-3'-phospho-pyridoxamine derivative.

18. The kit of claim 13 wherein RpX is a nucleoside-3'-phospho-thiamine derivative.

19. The kit of claim 13 wherein RpX is a nucleoside-3'-phospho-1,2-dioxetane derivative.

20. The kit of claim 13 wherein said detection system comprises a dehydrogenase, a diaphorase, and a tetrazolium compound.

21. The kit of claim 13 wherein said detection system comprises an apoenzyme.

22. The kit of claim 13 wherein said detection system comprises a phosphatase.

Figure 1

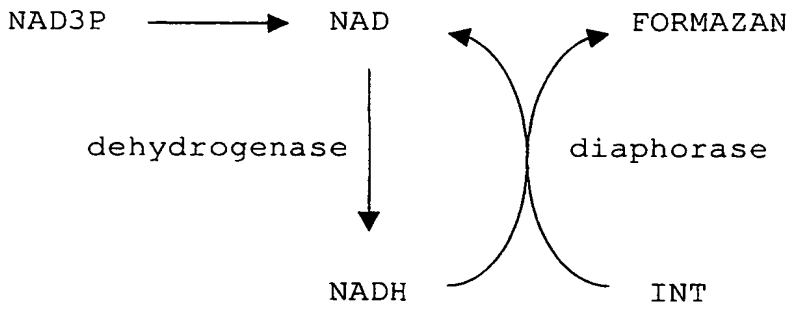


Figure 2

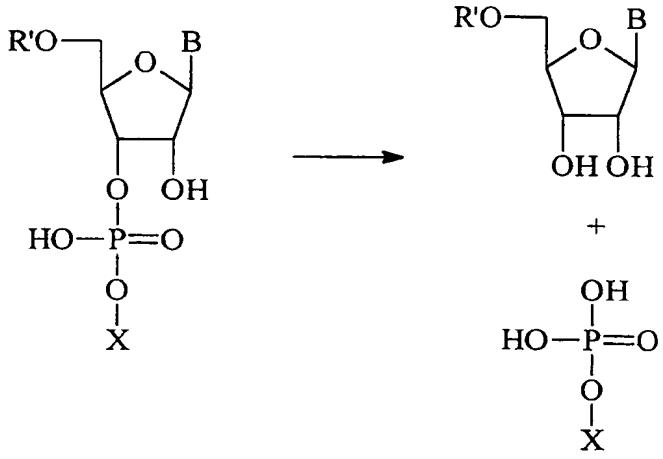


Figure 3

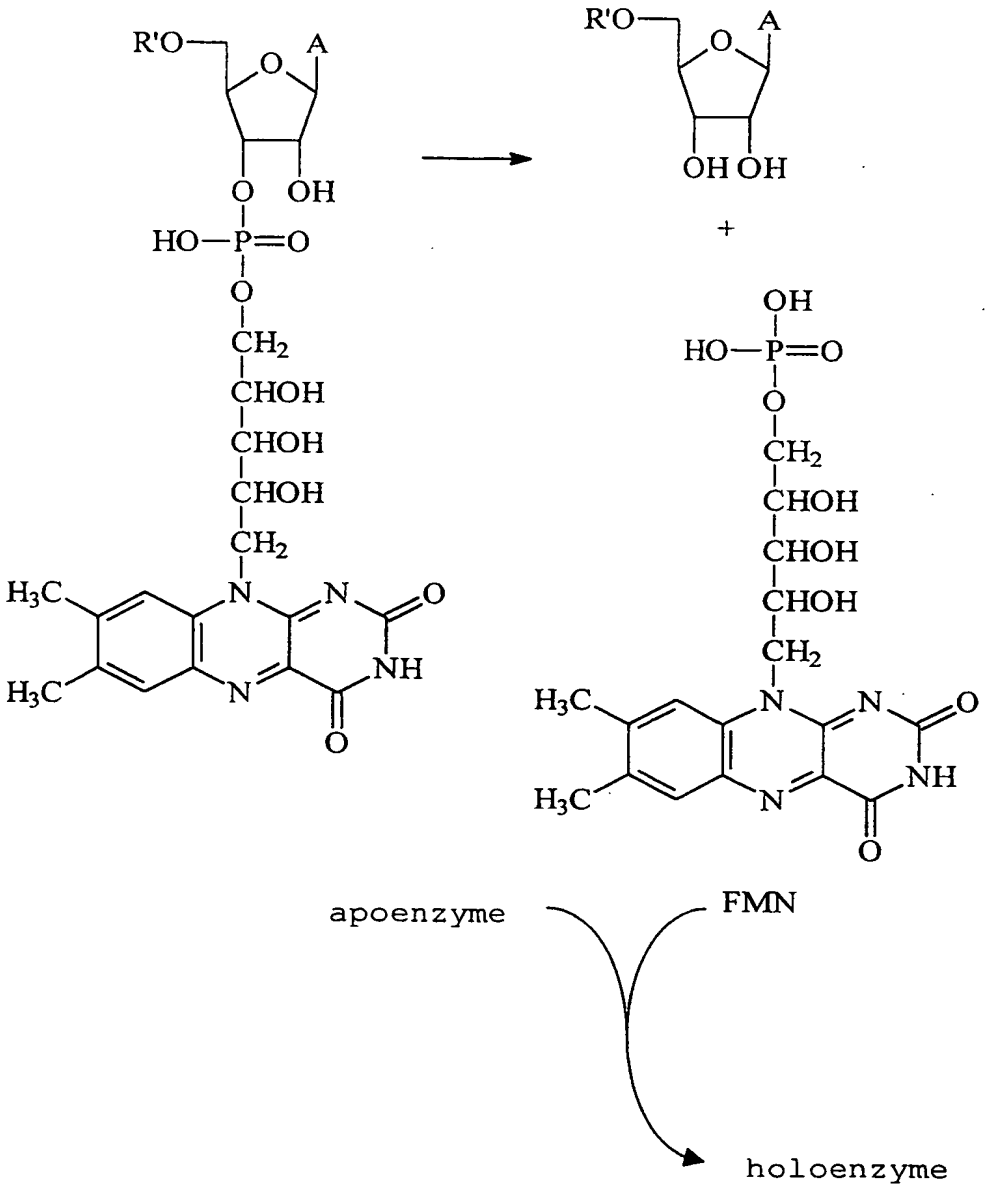

R'O
A
O
OH
HO—P=O
O
CH2
CHOH
CHOH
CHOH
CH2
H3C
H3C
N
N
O
NH
N
O
R'O
A
O
OH OH
+
OH
HO—P=O
O
CH2
CHOH
CHOH
CHOH
CH2
H3C
H3C
N
N
O
NH
N
O
apoenzyme
FMN
holoenzyme

Figure 4

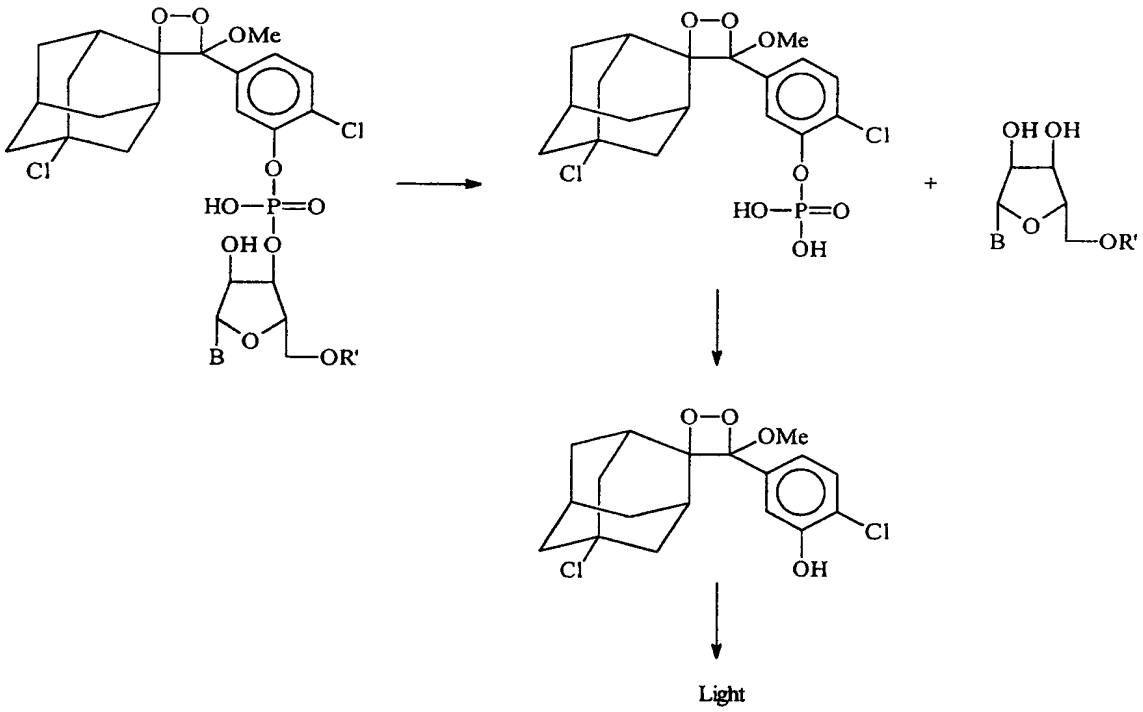


Figure 5

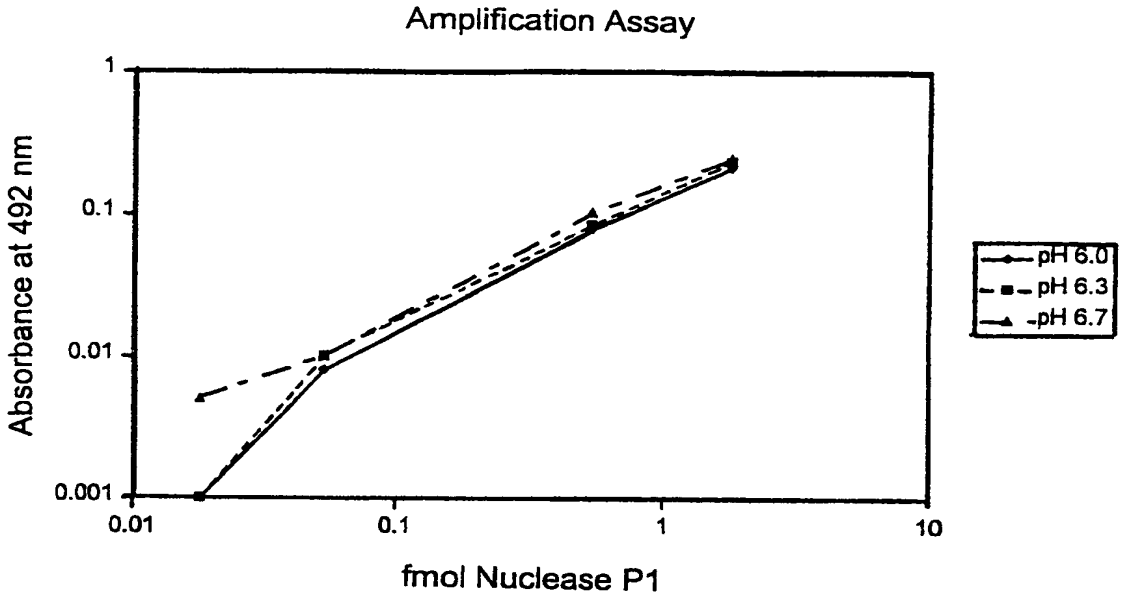
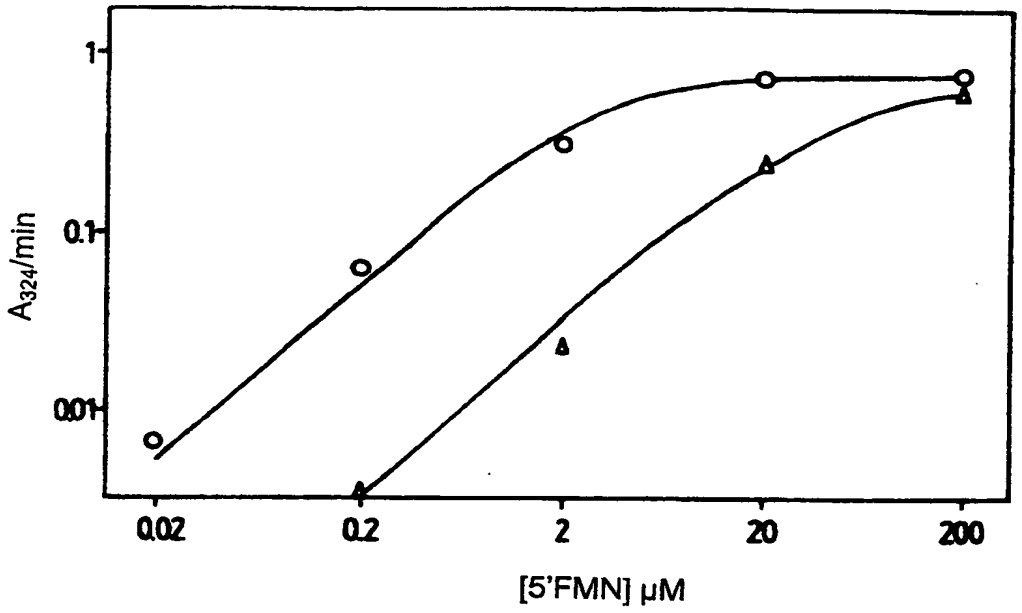

Amplification Assay
Absorbance at 492 nm
1
0.1
0.01
0.001
0.01
0.1
1
10
fmol Nuclease P1
pH 6.0
pH 6.3
pH 6.7

Figure 6



PATENT COOPERATION TREATY

PCT

S.R.

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
PA 3442 PCT INT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 00606	21/02/2000	20/02/1999
Applicant		
HARBRON, Stuart		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00606

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 156 641 A (UNIV LONDON) 2 October 1985 (1985-10-02) cited in the application page 3, line 27 -page 6, line 2; claims	1-6,9, 13,15, 16,18, 20,21
X	KARN R.C. ET AL: "A positive zymogram method for ribonuclease." ANALYTICAL BIOCHEMISTRY, (1979) 96/2 (464-468). CODEN: ANBCA2, XP000916155 the whole document	1,4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 July 2000	26/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Luzzatto, E

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KOLE, R. ET AL: "Specific colorimetric and cytochemical substrate for ribonuclease T2:adenosine-3'-(.alpha.-naphthylphosphate)" BIOCHIM. BIOPHYS. ACTA (1972), 289(2), 323-30 , XP000916205 the whole document	1,4,13
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A	HARBRON S ET AL: "AMPLIFIED ASSAY OF ALKALINE PHOSPHATASE USING FLAVINADENINE DINUCLEOTIDE PHOSPHATE AS SUBSTRATE" ANALYTICAL BIOCHEMISTRY, 1 October 1992 (1992-10-01), XP002072948 cited in the application the whole document	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No
PCT/GB 00/00606

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